Molecular Microbiology (2006) **61**(1), 76–88
doi:10.1111/j.1365-2958.2006.05215.x
First published online 1 June 2006

The *Aspergillus nidulans* F-box protein GrrA links SCF activity to meiosis

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Summary

Cellular differentiation relies on precise and controlled means of gene expression that act on several levels to ensure a flexible and defined spatio-temporal expression of a given gene product. In our aim to identify transcripts enriched during fruiting body formation of the homothallic ascomycete *Aspergillus* (*Emericella*) *nidulans*, the *grrA* gene could be identified in a negative subtraction hybridization screening procedure. It encodes a protein similar to fungal F-box proteins, which function as substrate receptors for ubiquitin ligases, and that is highly related to the *Saccharomyces cerevisiae* regulatory protein Grr1p. Expression studies confirmed induction of *grrA* transcription and expression of its gene product during cleistothecial development of *A. nidulans*. Functional complementation of a yeast *grr1Δ* mutant was achieved by overexpression of the *grrA* coding sequence. A *grrAΔ* deletion mutant resembles the wild-type in hyphal growth, asexual sporulation, Hülle cell formation or development of asci-containing cleistothecia, but is unable to produce mature ascospores due to a block in meiosis as demonstrated by cytological staining of cleistothecial contents. Our results specify a particular involvement of the E3 ubiquitin ligase SCF^{GrrA} in meiosis and sexual spore formation of an ascomyceteous fungus and shed light on the diverse functions of ubiquitin-proteasome-mediated protein degradation in eukaryotic development.

Introduction

Multicellular organisms accomplish a plethora of differentiation programmes in response to a variety of environmental inputs. One common basis for the generation of differentiated cell types is the precise interplay of regulation of gene expression and protein degradation in a spatial and temporal manner to result in defined levels of specific gene products. To achieve this objective, several modes of regulation can be classified such as regulation of transcription initiation, mRNA location and stability, or turnover rates of gene products. Besides transcriptional regulation, much attention has been drawn in recent years to the stability of proteins as a mode of regulation. Synthesis and degradation of a given gene product determine for what period of time or particularly when during the cell cycle the protein is present and able to fulfil its cellular tasks. This turnover of proteins, which is mediated by the covalent attachment of small modifiers such as ubiquitin followed by destruction in the proteasome, has been identified as a general matter in gene expression regulation. Especially E3 ubiquitin ligase activities, like the anaphase-promoting complex/cyclosome (APC/C) or the Skp/Cullin/F-box (SCF) complex, have emerged as vital regulatory focal points for fundamental processes like the eukaryotic cell cycle, meiosis, or development (Peters, 1998; Deshaies, 1999; Morgan, 1999; Vodermaier, 2004).

Filamentous fungi have come forward as simple and suitable eukaryotic model organisms in studying less complex differentiation courses such as hyphal growth or the production of reproductive structures that eventually result in dormant spores (Borkovich *et al*., 2004). In *Aspergilli*, in particular the latter process has been scrutinized thoroughly, because of the fact that the conidiophore, which produces the asexual spores and actually determines the genus morphologically, develops in a highly precise and defined manner with respect to induction and timing of the developmental process (Adams *et al*., 1998). In recent years, development of fruiting bodies accompanied by the generation of sexual spores has emerged as a point of interest for the ascomyceteous representative of the genus, *Aspergillus* (*Emericella*) *nidulans* (Champe *et al*., 1994). Here, ascogeneous hyphae fuse to eventually give rise to a large and complex structure, termed the cleistothecium. A first sign of fruiting body development is the appearance of supportive, thick-walled Hülle cells to form the so-called nests. Within these, immature fruiting bodies, primordia, develop that

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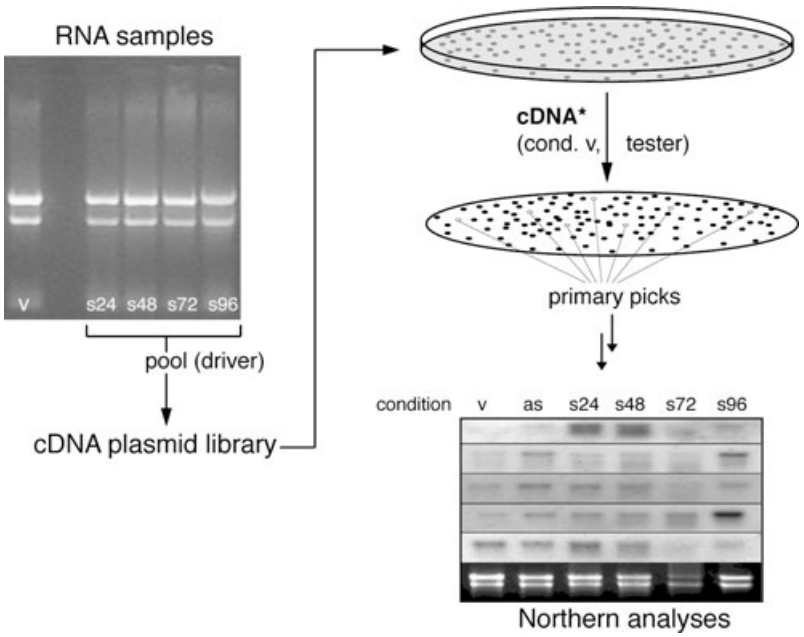

Fig. 1. Rationale of negative subtractive hybridization screening procedure. Pools of mRNA representing the transcriptomes of several time points during sexual development of *A. nidulans* (s24, s48, s72 and s96) were pooled and converted to one cDNA library maintained in bacterial cells. Aliquots of this library were regrown and evenly distributed on appropriate media, from which filter lifts were carried out. Samples derived from the RNA population that represents the tester condition 'vegetative growth' (v) were converted to labelled cDNA and hybridized to the immobilized filter probes. Primary picks that do not yield a signal are likely to be specific for one of the driver conditions but are absent under vegetative growth conditions. Further analyses and eventually Northern hybridizations confirmed differential expression of candidate transcripts.

eventually ripen to form a dark-pigmented, melanized spherical shell that contains thousands of asci with eight bi-nucleate, haploid ascospores each (Sohn and Yoon, 2002). Numerous environmental or genetic factors have been identified over the last decade to influence either of the cellular programme – nest formation, cleistothecia development, or ascosporogenesis (Braus *et al*., 2002), but only few reports have elucidated the developmental programme of ascospore formation in *A. nidulans* explicitly. By random sequencing of expressed sequence tags (ESTs) from a stage-specific cDNA library, Chae and coworkers isolated a group of sexual structure-specific transcripts, among them that of the ribosomal protein L16A-encoding gene *rpl16a* (Lee *et al*., 1996; Jeong *et al*., 2000). Moreover recently, an array of 18 ascosporogenous mutant strains clustering to 15 complementation groups was generated by random UV mutagenesis (Swart *et al*., 2001). However, the only mutation to date that has been precisely identified to impair proper ascospore formation in *A. nidulans* is the deletion of the α-tubulin-encoding gene *tubB* (Kirk and Morris, 1991); a corresponding mutant strain is unable to produce ascospores, which apparently results from a block prior to the first meiotic division, whereas asci formation or fruiting bodies' development is not impaired.

We here demonstrate that the SCF substrate adaptor protein GrrA of *A. nidulans* is specifically required for ascospore formation in this homothallic ascomycete and we therefore deduce an unexpected role for SCF-mediated protein ubiquitylation in fungal meiosis.

Results

Negative subtraction hybridization identifies the A. nidulans grrA *transcript to be expressed specifically during fruiting body development*

The identification of genes that are differentially expressed in the course of a cellular process provides hints about the regulatory circuits underlying this route. In our aim to analyse the transcriptional programme that determines fruiting body formation of the ascomycete *A. nidulans*, we made use of a simple but straightforward approach as it had been described recently (Ray *et al*., 2004). In this so-called negative subtractive hybridization (NSH) procedure, transcripts that are present in the mRNA pools derived from one or several cellular states but are absent in another specific one are identified by hybridizing representative cDNA pools to each other. There, the former cDNA pool (driver) is immobilized on a substrate such as a membrane, and the tester pool is labelled for identification of hybridizing events. The absence of a hybridization signal indicates that the corresponding transcript is present in the driver pool but absent in the tester pool, which points to the fact that the encoding gene is expressed under one of the driver conditions but not in the tested one. Primary cDNA picks are then checked subsequently against each of the driver condition and finally validated for differential expression by Northern analyses (Fig. 1).

As initial step of our studies a comprehensive cDNA library was generated in form of a plasmid pool. For that purpose, RNA was isolated from the *A. nidulans* isolate

FGSC A4 (Glasgow wild-type) after induction of vegetative cultures for sexual differentiation. After transferring pregrown mycelia and sealing the plates for restriction of aeration, incubation was carried out in the absence of light. Consequently, synchronous development of Hülle cells and cleistothecia that harboured a large number of asci containing eight binucleate ascospores each took place without asexual sporulation to occur. Samples were harvested and processed after defined periods of time, and from the pool of prepared RNA, cDNA was generated to be eventually ligated into a plasmid vector backbone (see *Experimental procedures* for details). Pools of *Escherichia coli* cells replicating the pCNS4 cDNA library plasmids were allowed to grow on standard media supplemented with the antibiotic carbenicillin to maintain the library plasmids. These colonies were lifted on membranes, on which nucleic acids were immobilized after the cells' lysis. In parallel, freshly prepared RNA samples from vegetative FGSC A4 cultures were labelled by incorporation of radio-nucleotides during reverse transcription from an oligo(dT) primer. Hybridizations and washing steps according to standard protocols finally resulted in autoradiographies from which non-hybridizing library colonies could be identified by tracing them back to their position on the initial plate. From approximately 6000 colonies, a fraction of 300 were identified that did not yield a signal in the hybridization process. Subsequent hybridizations of this preliminary array of clones with labelled samples from sexually induced wild-type cultures narrowed this pool of primary picks, from which four colonies were chosen as representative output for the NSH screening procedure. Northern analyses employing the corresponding cDNAs as probe templates confirmed the expression patterns during the developmental course of cleistothecia formation as expected.

One of the differentially expressed transcripts was scrutinized further. The corresponding cDNA insert of the isolated library plasmid pME3019 was sequenced to completion, and based on this sequence information a HpaI/NheI 8.7 kb genomic DNA fragment of FGSC A4 could be isolated from a size-fractionated library (pME3023). As judged from its sequence, pME3019 contains an incomplete cDNA insert as no complete coding region could be deduced from its sequence. To obtain the full-length transcript's copy, the 5′ region of the cDNA was amplified from the pCNS4 library employing gene-specific oligonucleotides. A defined polymerase chain reaction (PCR) product could be isolated and sequence analysis confirmed that it completes the formerly isolated cDNA to yield a full coding sequence. Combination of this amplified 5′ region with the C-terminal part of pME3019 yielded pME3020 containing the complete cDNA open reading frame. By comparison with its genomic locus, the architecture of the identified gene could be determined (Fig. 2A). Three exons of 732 bp, 685 bp and 342 bp that are interrupted by two introns 49 bp and 57 bp in size define the coding region, which has the capacity to encode a 65 kDa polypeptide. Comparison of this deduced amino acid sequence revealed a high degree of similarity of the proposed gene product with a counterpart encoded in the genome of *Neurospora crassa* (GenBank accession no. CAD21405). Furthermore, both orthologues share striking similarities to the F-box protein Grr1p of the baker's yeast *Saccharomyces cerevisiae* (accession no. NP_012623). In particular, a stretch of 63 amino acids could be identified in the gene product's primary sequence that resembles the evolutionary conserved F-box motif, followed by a domain of 383 residues that is characterized by the accumulation of leucine-rich repeats (LRRs) (Fig. 2B and C). Based on this high degree of similarity to its yeast counterpart, the identified *A. nidulans* gene was designated as *grrA* (accession no. DQ309327). The high degree of conservation of GrrA is further supported by comparison with fungal sequence databases: submitting the yeast Grr1p sequence to the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul *et al*., 1997) returns the hypothetical protein AN4200.2 as closest related sequence deduced from the *A. nidulans* genome sequence, which is, apart from annotation errors, identical to GrrA.

The grrA *gene product is a functional orthologue of yeast Grr1p*

To test the hypothesis that the identified *A. nidulans* gene encodes a true orthologue of the *S. cerevisiae* F-box protein Grr1p, constructs were made to express the *grrA* gene product in a yeast *grr1Δ* background. For that purpose, the complete *grrA* coding sequence was inserted into an expression module consisting of the strong yeast *MET25* promoter and the termination region of the *CYC1* gene to be located in an autonomously replicating plasmid. Two expression plasmids were constructed (pME3021 and pME3022), which differ in their copy number within the host. The haploid *grr1::kanMX* strain Y16902 as created in the systematic EUROSCARF deletion mutant series served as recipient and transformants were selected for the presence of either plasmid. Inspection of the transformants' pools revealed complementation capacities of the heterologous gene. Yeast *grr1*$^{-}$ mutants display a variety of phenotypes, among them an aberrant morphology: haploid strains devoid of Grr1p appear elongated and display a cytokinesis defect due to improper stabilization of the G1 cyclins Cln1 and Cln2 (Flick and Johnston, 1991; Barral *et al*., 1995). As a consequence, mother and daughter cells remain attached to each other after the budding process,

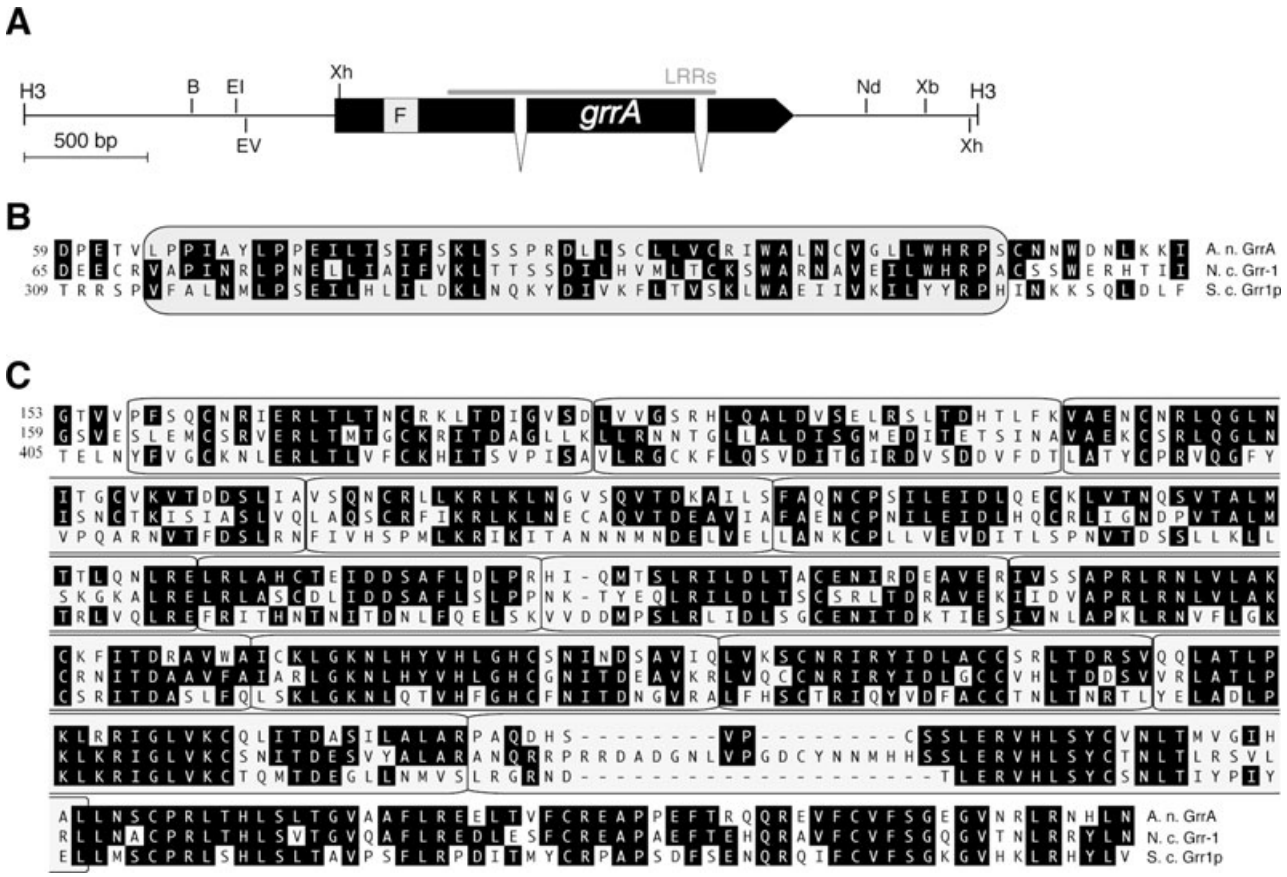

Fig. 2. The *grrA* gene product represents an F-box protein that is conserved among fungi.
A. Architecture of the genomic locus encompassing the *grrA* gene of *A. nidulans*. Exons are depicted in black; the conserved F-box motif (F) is indicated as grey square, the region spanning LRRs as dark-grey bar. Recognition sites of common restriction endonucleases are given as H3 (HindIII), EI (EcoRI), Xh (XhoI), Xb (XbaI), EV (EcoRV) and B (BstEII).
B. Local alignment of the conserved F-box region (grey rectangle) of GrrA with counterparts from *N. crassa* (*N. c.* Grr-1) and *S. cerevisiae* (*S. c.* Grr1p). Positions in the primary structure of the respective proteins are indicated and conserved residues are boxed in black.
C. Alignment of GrrA, Grr-1 and Grr1p LRRs as deduced from the primary structures. Repetitive modules are specified by light-grey rectangles and conserved residues are boxed in black.

resembling a pseudohyphal growth mode. When expressed at intermediate levels, GrrA partially rescues this particular phenotype as a high proportion of transformed *grr1Δ* cells display a round and separated cell form (Fig. 3A). When expressed from the multicopy construct, these complementation capacities appeared even more pronounced and here the majority of cells resemble the wild-type morphology. Conclusively, the *grrA* gene product is able to rescue the morphology defect of a haploid *S. cerevisiae* strain lacking Grr1p. To characterize this complementation ability of *grrA* further, the transformed yeast cells were tested for the regulation of genes involved in hexose transport and glycolysis upon a shift from the carbon source galactose to the fermentable sugar glucose (Fig. 3B). RNA samples prepared from shifted cultures were tested for steady-state levels of the transcripts *HXK1* and *HXT3* by Northern analyses. As judged from the transcription patterns of the wild-type control strain BY4742, the GrrA protein fully restores the transcriptional repression or induction of each reporter transcript: whereas in the wild-type background almost no hexokinase-encoding *HXK1* transcript is detectable upon the galactose-to-glucose shift, the *grr1Δ* mutant strain expresses the transcript at high levels when grown in the presence of glucose, and this phenotype was restored to the wild-type in strains carrying the ^{p}*MET25::grrA::CYC1*^{t} expression plasmid. Reciprocally, transcript levels of the hexose transporter-encoding *HXT3* gene are almost absent in the *grr1Δ* background when grown in galactose or glucose, and either in the wild-type or *grrA*-expressing strains a clear signal could be detected when cells were transferred to glucose. In summary, these results clearly indicate that the *A. nidulans*-encoded GrrA represents a well-conserved, functional orthologue of the *S. cerevisiae* F-box protein Grr1p.

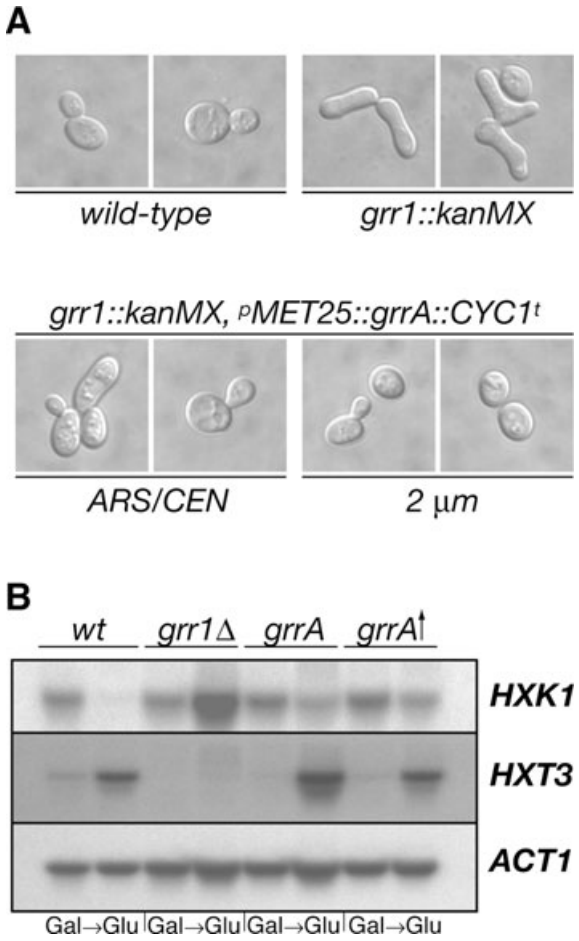

Fig. 3. GrrA is a functional orthologue of *S. cerevisiae* Grr1p.
A. Functional complementation of the yeast *grr1Δ* morphology phenotype is achieved by expression of *A. nidulans grrA*. Low-copy expression of *grrA* in the haploid yeast *grrA::kanMX* recipient from an *ARS/CEN* plasmid restores round morphological appearance, which is even more pronounced when expressing the *grrA* cDNA from a multicopy 2 μm episome.
B. *Aspergillus nidulans* GrrA can substitute for *S. cerevisiae* Grr1p in the transcriptional reprogramming of yeast strains upon a shift from galactose to glucose (Gal → Glu). Steady-state transcript levels of the *HXK1* gene coding for a hexose kinase and the hexose transporter-encoding gene *HXT3* were determined by Northern analyses, and constitutive expression of the actin-encoding *ACT1* transcript served as internal control. Expression of the *A. nidulans* orthologue restores the expression patterns for both reporter transcripts as exhibited by the wild-type strain.

Expression of GrrA is particularly induced during ascospore formation within the A. nidulans *cleistothecium*

After identifying the *grrA* transcript in the NSH screening procedure to be specifically expressed during fruiting body development of *A. nidulans*, we analysed the expression pattern of the *grrA* gene and its encoded protein in more detail. Initially, RNA samples derived from a vegetative culture or specifically induced mycelia of wild-type strain A4 were prepared after defined time periods of the developmental course to carry out a precise Northern analysis (Fig. 4A). As judged from the hybridization signals, the *grrA* gene is transcribed at low levels during hyphal growth in liquid culture, and this basal level is slightly elevated when asexual sporulation is induced. Following the developmental sequence of cleistothecia development, which is accompanied by the differentiation of Hülle cells and eventually ascospore formation, a significant rise in *grrA* transcript levels can be detected in Northern analyses. The signal's increase coincides with the intermediary phase of the developmental pathway, starting after 72 h post induction to result in peak levels at approximately 120 h after the onset of differentiation. To monitor expression and localization of the GrrA protein, the gene product was expressed as green fluorescent protein (GFP) fusion protein. For that purpose, the coding sequence of the fluorescent tag was fused to the N-terminus of the *grrA* coding sequence separated by a GA_5 repeat linker region. The resulting allele, with its expression driven by the constitutive *gpdA* and the native *grrA* promoter, respectively, was used to transform a *grrA* wild-type recipient (AGB152) as well as to reconstitute a *grrAΔ* deletion mutant strain (AGB259, see below). The resulting strains AGB260 and AGB261 were able to form ascospores, demonstrating that the GFP-GA_5-GrrA construct is functional in complementing the *grrA* null phenotype. The chimeric construct was found to reside in the cytoplasm of hyphal cells when expressed from the constitutive promoter (Fig. 4B). When monitoring fluorescence in strain AGB261, no clear signal could be observed in structures such as vegetative mycelium or conidiophores (not shown). However, when inspecting the cellular contents of immature cleistothecia, a distinct fluorescence in the cytoplasm of ascogenous cells was evident (Fig. 4C). Within these cells, defined areas that resembled immature ascospores were excluded from the staining. Other cells within the cleistothecia such as ascogenous hyphae, croziers or mature asci that contain octades of ascospores did not exhibit any specific fluorescence. Altogether, these data indicate a highly specific spatio-temporal expression pattern of the *grrA* gene product, mediated by increased transcription at the onset of sexual differentiation and a restricted expression within ascocarp cells.

Deletion of grrA *impairs ascosporogenesis in* A. nidulans

To assess any cellular function of the *A. nidulans* GrrA protein, a deletion mutant was generated in a genetic wild-type background. For that purpose, a suitable replacement cassette was constructed employing the dominant *Aspergillus oryzae* marker gene *ptrA* to make use of a directional ligation-mediated approach (see

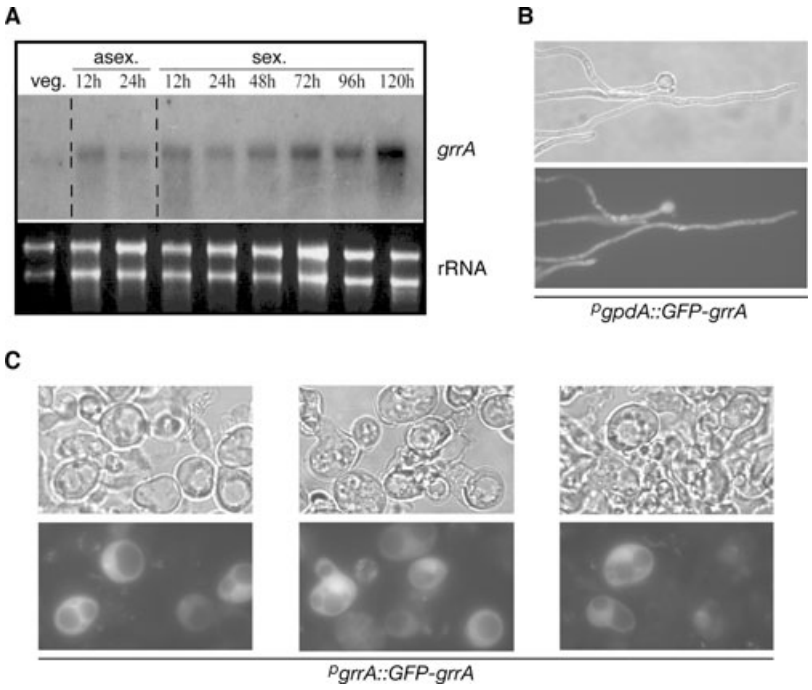

Fig. 4. Expression of GrrA is specifically increased during cleistothecia development within ascogenous cells.
A. Steady-state *grrA* transcript levels as determined by Northern analyses from sexually induced *A. nidulans* cultures are elevated during fruiting body development. Vegetative cultures (veg.) of *A. nidulans* wild-type isolate FGSC A4 were transferred to solid media, and developmental courses of asexual sporulation (asex.) or cleistothecia formation accompanied by ascosporogenesis (sex.) were induced specifically for the periods of time as indicated before total RNA was prepared; bands of ethidium bromide-stained rRNA are given as loading control.
B. GrrA localizes to the cytoplasm. Phase-contrast and fluorescence images of vegetative mycelium from strain AGB260 (*PgpdA::gfp-grrA*; *grrA*) are shown, displaying cytoplasmic staining throughout the vegetative hypha.
C. GrrA is specifically expressed in ascus mother cells. The upper panel shows phase-contrast images from cleistothecial contents of strain AGB261 (*PgrrA::gfp-grrA*; *grrAΔ*) facing corresponding GFP fluorescence images. Samples were isolated from immature, 2–3 days old fruiting bodies. The representations demonstrate a well-defined staining of the cytoplasm of ascocarp cells, within which ascospore formation has been initiated, which reveals functionality of the chimeric fusion protein.

Experimental procedures for details). Transformation of the pME3025-derived *grrA::ptrA* fragment into the wild-type recipient FGSC A4 resulted in one transformant in which the endogenous locus had been replaced by the marker cassette as confirmed by comprehensive Southern analysis (Fig. 5A and B). This primary deletion mutant was back-crossed to its progenitor and from the resulting descendants one representative, AGB258, was characterized further. When compared with its wild-type ancestor on standard minimal medium supplemented with ammonium as nitrogen source and glucose as source of carbon, no apparent phenotype in growth, asexual conidiation or sexual development could be observed (Fig. 6A). This was also evident when comparing growth of both strains on the alternative carbon source galactose (data not shown). However, when opening one of the fruiting bodies derived from the *grrA::ptrA* mutant, a diffuse mass of cells could be detected and the complete lack of ascospores was evident (Fig. 6B). Plating the contents from several AGB258-derived cleistothecia on standard medium did not give rise to any colonies, indicating that no functional ascospores are formed in the fruiting bodies of the *grrAΔ* strain. Closer inspection of the developmental course of ascosporogenesis within wild-type and *grrAΔ* fruiting bodies was undertaken by staining the cleistothecial contents with acriflavin, a potent fluorescent dye for nuclei. Within normal cleistothecia the onset of ascospore formation is detectable after about 2 days by the appearance of crozier-like structures that contain diploid nuclei in the penultimate cell to give rise to an octade of red-pigmented ascospores within an ascus (Fig. 6C). In contrast, diffuse and bright areas of DNA could be stained within the croziers of the *grrA* deletion strain that disappeared as ascus development proceeded.

As deduced from these cytological examinations, an irreversible cessation at early ascospore development is evident that hints to a block in *A. nidulans* meiosis provoked by the absence of the GrrA F-box protein. This remarkable phenotype could be restored by cotransformation of the deletion strain AGB258 with a genomic fragment encompassing the *grrA* locus; among the transformants, a high proportion regained the ability to

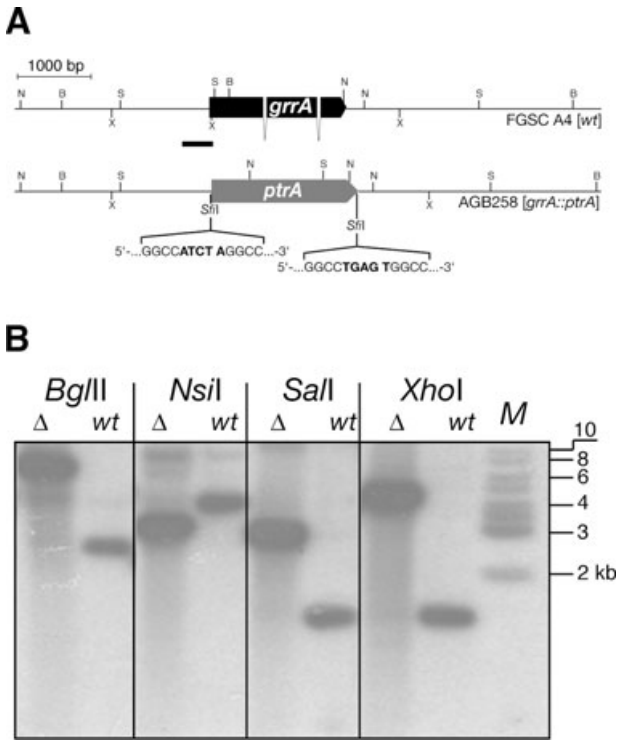

Fig. 5. Generation of a *grrA* deletion mutant strain.
A. Schematic representation of the *grrA* gene and the genomic locus after deletion by a *grrA::ptrA* replacement cassette. Positions of restriction endonuclease target sites are indicated for BglII (B), NsiI (N), SalI (S) and XhoI (X), the black bar specifies the region covered by the probe that was used for Southern analyses. Sequences of the asymmetric SfiI junctions that sandwich the *ptrA* resistance marker are indicated.
B. Autoradiography of Southern hybridization after digestion of genomic DNA from FGSC A4 (wt) and the *grrA::ptrA* deletion strain AGB258 (Δ). M specifies the DNA size standard with fragments' lengths given on the right-hand side.

form ascospores from which colonies could be regrown (not shown). Conclusively, the lack of ascospores as demonstrated by the appearance of empty asci within cleistothecia is solely attributed to the lack of the F-box protein GrrA. In agreement with this observation is the specific expression pattern of the *grrA* gene during fruiting body development at a time window during which ascospore formation predominantly occurs in *A. nidulans*. That this specific expression pattern is a strict prerequisite for accurate ascospore formation is a novel finding, which is validated by the fact that a chimeric GFP-GrrA fusion protein has to be expressed from the native *grrA* promoter to be functional, whereas the same construct expressed from the constitutive *gpdA* promoter (pME3028) is not able to rescue the *grrAΔ* mutant phenotype (data not shown). However, expression of this construct in a *grrA*$^{+}$ genetic background leads to ascospore formation, as it was observed for strain AGB260.

Accordingly, these data imply that the accurate action of a putative SCF^{GrrA} complex as E3 ubiquitin-ligating activity is a strict prerequisite for *A. nidulans* to fulfil meiosis and ascosporogenesis properly.

Discussion

We describe the identification and functional characterization of the SCF adaptor protein GrrA from the homothallic ascomycete *A. nidulans*. The *grrA* transcript was isolated in a screening procedure employing the negative subtraction hybridization approach. In our studies, the NSH screening procedure has turned out to be simple as well as straightforward, although the high proportion of background noise lowered the overall yield of transcripts specifically expressed during the condition tested.

As a validated clone from the NSH screening procedure a transcript with similarity to fungal F-box proteins was identified. F-box proteins serve as substrate receptors in cullin-ring ubiquitin ligases (CRLs), more precisely in SKP1, CUL1, F-box (SCF) proteins (for a recent review see Cardozo and Pagano, 2004). These conserved multisubunit protein complexes function as modular E3 ubiquitin ligases to promote ubiquitylation of numerous substrates, which targets them in the majority of cases for degradation by the 26S proteasome. The core of canonical SCF complexes is invariantly represented by a cullin protein (CUL1). Substrate receptors, which are recruited to this cullin by the SKP1 adaptor protein, vary immensely in number (Kipreos and Pagano, 2000). These F-box proteins are characterized by their domain architecture: the conserved F-box motif interacts with the adaptor and a second protein–protein interaction domain recruits the substrate for ubiquitin attachment. As deduced from *in silico* annotation studies (Madera *et al*., 2004), the *A. nidulans* genome has the capacity to encode at least 28 proteins that enclose an F-box domain. Numerous means of regulating the activity of SCF E3 ubiquitin ligases have been postulated, and one involves combinatorial control by differential expression of F-box protein components that compete for limiting amounts of the CUL1-SKP1 core complex (Patton *et al*., 1998). Our results of differential expression of GrrA clearly support this regulatory mechanism and imply the need for specific degradation of distinct SCF targets during the developmental route of ascospore formation. It will be of special interest to elucidate the regulatory circuit resulting in transcriptional induction of *grrA* expression, as transcription factors acting specifically in the course of this developmental path have not been identified to date.

A vast number of reports have demonstrated a role of the yeast SCF^{Grr1p} complex in several cellular processes,

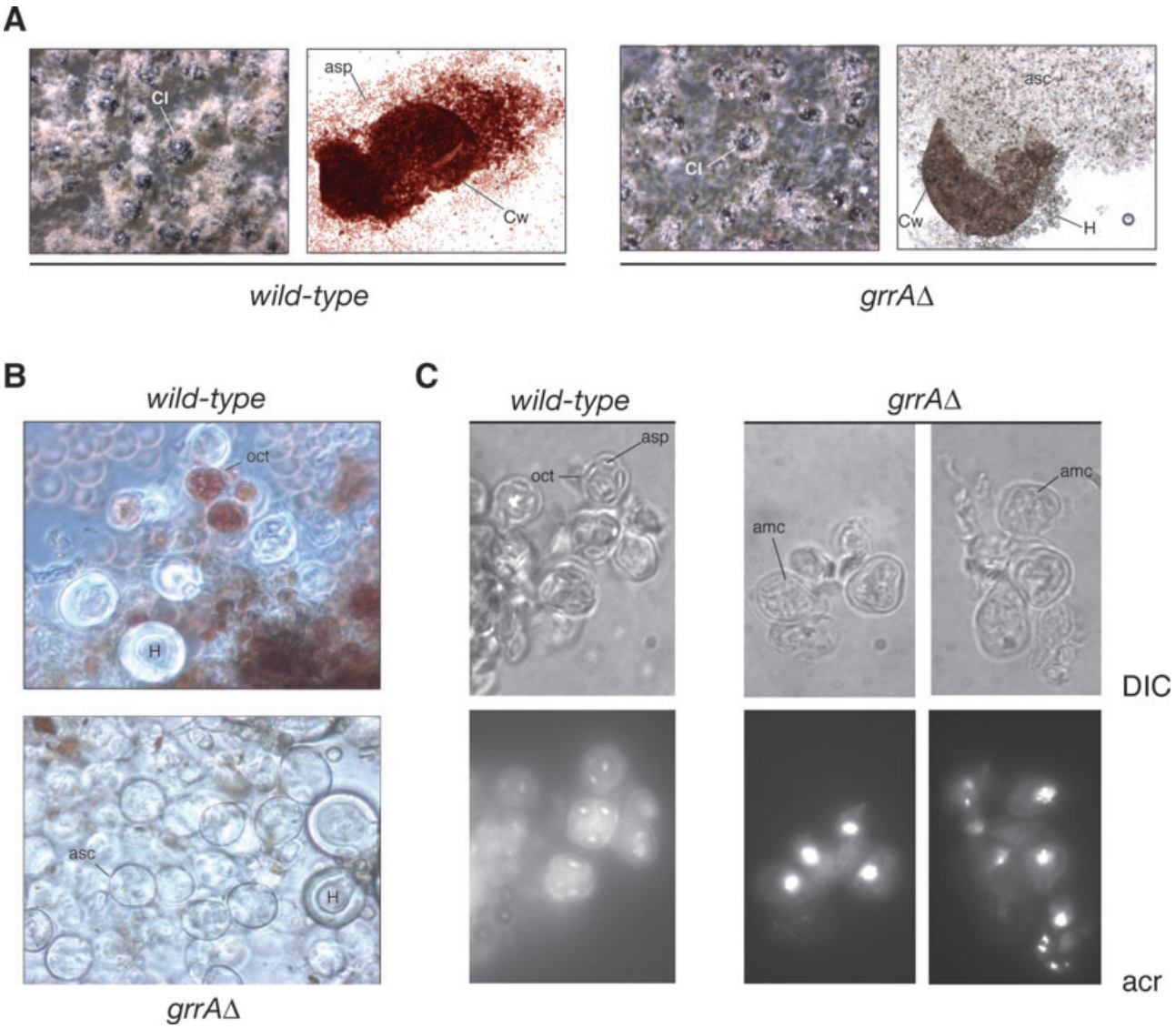

Fig. 6. Absence of GrrA impedes proper ascosporogenesis in *A. nidulans*. Phenotypical and cytological characterization was carried out to assess the impact of the *grrA*-encoded gene product on cellular aspects of *A. nidulans*.
A. Inspection of fruiting bodies (Cl) formed by the wild-type strain FGSC A4 (left) and the *grrAΔ* mutant strain (right) does not indicate significant differences with respect to Hülle cell (H) formation or formation of the cleistothecial walls (Cw). When cleistothecial contents are released by squeezing mature fruiting bodies, no ascospores but empty asci (asc) are released from *grrAΔ*-derived cleistothecia, in contrast to FGSC A4, from which masses of red-pigmented meiospores (asp) can be liberated.
B. Microscopic inspection of cleistothecial contents derived from FGSC A4 and AGB258 (*grrAΔ*). Whereas from the former strain mature octades of ascospores (oct) can be identified (top), only empty asci (asc) are found in the deletion mutant's fruiting bodies (bottom). Hülle cells (H) in the specimens stem from incomplete stripping of the cleistothecia.
C. Cytological staining with acriflavin (acr) indicates improper meiosis to occur in asci of the *grrAΔ* deletion mutant. Nuclei within octades (oct) of immature ascospores (asp) can be identified within wild-type asci (left), whereas diffuse masses of nucleic acids are stained within the ascus mother cells (amc) of AGB258 (right). Additional staining of multinucleate cells is likely to arise from croziers' tip or basal cells.

such as glucose sensing and catabolite repression, amino acid signalling, polarized bud growth, cytokinesis, the yeast cell cycle, or, most recently, retrograde signalling (Flick and Johnston, 1991; Li and Johnston, 1997; Blondel *et al*., 2000; Spielewoy *et al*., 2004; Liu *et al*., 2005; Schweitzer *et al*., 2005). Lately, Nielsen and coworkers have demonstrated a global role for Grr1p in the transcriptional response of *S. cerevisiae* on glucose repression (Westergaard *et al*., 2004; Eckert-Boulet *et al*., 2005), and Mth1p and Gis4p were identified in additional studies as prime targets for degradation to modulate the glucose response in yeast (Flick *et al*., 2003; La Rue *et al*., 2005). In our analyses of the *A. nidulans grrAΔ* strain AGB258, we were not able to detect any obvious phenotype asso-

ciated with carbon metabolism, which might indicate a more specific and less pleiotrophic role of the F-box protein in the life cycle of this filamentous fungus. The fact that GrrA is able to complement yeast deletion phenotypes such as derepression of hexose transporters upon a shift from galactose to glucose accounts for a conserved function of GrrA in the endogenous host; however, further studies will have to address the actual cellular function of the *grrA* gene product in *A. nidulans*. The only predominant phenotype associated with the lack of GrrA is the inability of a corresponding *A. nidulans* mutant to form ascospspores, whereas Hülle cell formation and cleistothecia development appear as wild-type. Cytological staining hints towards a block in meiosis that impairs the maturation of ascospores within the asci. The *GRR1* gene product of *S. cerevisiae* has a well-characterized function in the cell cycle of this yeast via degradation of the G1 cyclins Cln1p and Cln2p, and recently a role in meiosis could be established as well. In the baker's yeast glucose metabolism and reproductive modes are closely linked: the cell cycle is stimulated by glucose whereas sporulation is accurately inhibited by this hexose. This block in meiosis is mediated by Sic1p, a cyclin-dependent kinase inhibitor, and its regulator Ime2p, the destruction of which is mediated through SCF^{Grr1p} (Purnapatre *et al*., 2005). Diploid *grr1Δ/grr1Δ* mutants form so-called flower colonies that are indicative of meiosis when grown on 2% glucose (Purnapatre and Honigberg, 2002), supporting the role of Grr1p as repressor of meiosis in the presence of glucose. For *A. nidulans* such a close correlation of glucose concentration and sexual sporulation has not been established as this homothallic fungus forms efficiently fruiting bodies and ascospores on rich media that are supplemented with glucose. The yeast orthologue Grr1p was moreover found to influence ascospore formation positively, as *grr1Δ/grr1Δ* mutants display non-dysjunction in meiosis I to result in chromosome missegregation (Marston *et al*., 2004). However, *S. cerevisiae grr1Δ/grr1Δ* mutants form asci that contain meiotic spores, although these are distinct from wild-type asci in terms of packaging, ascospore numbers and spore viability (Purnapatre *et al*., 2005). The phenotype that is associated with a lack of the orthologue in *A. nidulans* results in the complete absence of ascopsores within cleistothecia of such mutant strains. Furthermore, meiosis appears to be impaired at an early stage and therefore blocks subsequent ascospore development but not asci formation. Accordingly, our results specify GrrA of *A. nidulans* as a positive-acting factor of meiosis and support a link between SCF-mediated protein degradation and meiosis preceding ascospore formation. The cytological appearance of the *grrAΔ* mutant strain AGB258 is similar to mutants blocked at meiotic prophase as they were characterized in the mutant collection of Swart *et al*. (2001). Furthermore, the *grrAΔ* mutant strain closely resembles the mutant phenotype of a *tubBΔ* deletion strain by the appearance of one nuclear mass within the ascus mother cell (Kirk and Morris, 1991). Accordingly, the phenotype of an *A. nidulans* mutant impaired in SCF^{GrrA} function by lack of the substrate receptor resembles a block in early meiosis. This is substantiated by the restricted localization of GrrA within the developing cleistothecium: the exclusive expression within the cytoplasm of the ascogenous cells coincides with ascopsore formation, which relies on proper meiosis to occur.

In preliminary efforts to yield further insights about the cellular tasks of GrrA, we attempted to isolate *A. nidulans* double mutants impaired in COP9 signalosome (CSN) function (Busch *et al*., 2003) and lacking this F-box protein. When crossing the *grrAΔ* strain AGB259 to the *csnDΔ* mutant AGB195, acospores could be readily isolated from cleistothecia of the heterokaryon – however, from those no descendant carrying both genetic lesions could be identified (our unpublished results). This points to a synthetic lethality of impaired CSN and GrrA functions. Although both factors are phenotypically assigned to sexual propagation of *A. nidulans*, this putative genetic interaction implies a more general requirement of the CSN- and GrrA-associated cellular functions in the life cycle of this fungus.

In summary, our studies reveal an unprecedented role of the F-box substrate receptor GrrA and presumably its corresponding SCF^{GrrA} complex in fungal meiosis. Activity of this ubiquitin-conjugating activity is strictly required at an early stage of meiosis, but the precise mode of action and the substrate(s) targeted for ubiquitylation remain to be identified.

Experimental procedures

Strains, media and growth conditions

Fungal strains used throughout this study are listed in Table 1. The prototrophic FGSC A4 strain as obtained from the Fungal Genetics Stock Center (Kansas City, MS, USA) served as *A. nidulans* wild-type isolate. *S. cerevisiae* strains were ordered from the systematic deletion collection EUROSCARF (Brachmann *et al*., 1998) (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/). *E. coli* DH5α (Woodcock *et al*., 1989) and SURE® (Stratagene) strains were used for preparation of plasmid DNA and were propagated in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) in the presence of ampicillin at 100 μg ml^{-1}. Minimal medium (MM: 0.52 g l^{-1} KCl, 0.52 g l^{-1} $MgSO_4$, 1.52 g l^{-1} KH_2PO_4, 0.1% trace element solution, pH 6.5) was used for growth of *Aspergillus* strains (Käfer, 1965), supplemented with pyrithiamine (0.1 μg ml^{-1}) or uracil/uridine (5 mM) as required. As carbon source 1% D-glucose was used, and as nitrogen source 10 mM ammonium was supplemented in form of its tartrate salt. Sexually developing cultures to produce the pCNS4 cDNA plasmid library had been propagated as vegetative cultures in liquid

Table 1. Fungal strains.

Strain	Genotype		Reference
Aspergillus nidulans			
FGSC A4	Glasgow wild-type		Fungal Genetics Stock Center
AGB152	*pyroA4*; *pyrG89*		Busch *et al*. (2003)
AGB195	*csnD::pyr-4*; *pyrG89*; *pyroA4*		Busch *et al*. (2003)
AGB258	*grrA::ptrA*	[FGSC A4 + pME3025 (BglII/StuI)]	This study
AGB259	*grrA::ptrA*; *pyrG89*	[AGB258 × AGB195]	This study
AGB260	*^{p}gpdA::gfp-GA$_5$ -grrA*, *A.f. pyrG*; *pyrG89*	[AGB152 + pME3028]	This study
AGB261	*^{p}grrA::gfp-GA$_5$ -grrA*, *A.f. pyrG*; *pyrG89*; *grrA::ptrA*	[AGB259 + pME3027]	This study
Saccharomyces cerevisiae			
BY4742	*MATα*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*		EUROSCARF collection
Y16902	*MATα*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*; *YJR090c::kanMX4*		EUROSCARF collection

MM + CA medium (Han *et al*., 1990) containing 1% glucose as carbon source and 0.1% sodium nitrate, 0.1% casein hydrolysate as sources of nitrogen, and were transferred to solid MM + CA after 18 h of growth. Plates were sealed with parafilm and wrapped with aluminium foil in order to induce fruiting body formation specifically. *S. cerevisiae* strains were grown on supplemented minimal media essentially as described by Guthrie and Fink (1991).

Transformation procedures

Escherichia coli cells were either transformed after calcium/manganese treatment (Hanahan *et al*., 1991) or made competent for electroporation (Taketo, 1988) with a BIO-RAD GenePulser at 2.5 kV in 0.2 cm cuvettes. *A. nidulans* was transformed by polyethylene glycol-mediated fusion of protoplasts following the procedure of Punt and van den Hondel (1992). Yeast recipient strains were transformed with plasmids according to the protocol of Elble (1992) and transformants were selected on dropout media.

Recombinant DNA procedures and hybridization techniques

Standard protocols of recombinant DNA technology were carried out according to Sambrook *et al*. (1989). *Taq* and *Pfu* polymerases were generally used in PCRs (Saiki *et al*., 1985) and essential cloning steps were verified by sequencing on an ABI PRISM 310 capillary sequencer. *A. nidulans* genomic DNA was prepared from ground mycelia according to Kolar *et al*. (1988) and Southern analyses were carried out as described (Southern, 1975). Samples of total RNA were isolated from *A. nidulans* strains using the TRIzol reagent of Invitrogen or from *S. cerevisisae* strains according to Cross and Tinkelenberg (1991). Northern hybridizations were performed following the protocols listed by Brown and Mackey (1997). Random primed labelling of hybridization probes (Feinberg and Vogelstein, 1983) was achieved with the STRATAGENE PRIME-IT® II kit in the presence of [α-^{32}P]-dATP, and washed membranes were exposed to Kodak X-OMAT films to generate autoradiographies. Sequence analyses were carried out using the LASERGENE Biocomputing software package from DNASTAR, alignments were created by the Lipman–Pearson Method (Lipman and Pearson, 1985).

cDNA library screening by NSH (Ray et al.*, 2004)*

To create a cDNA library that is enriched for transcript copies specific for fruiting body formation of *A. nidulans*, total RNAs were prepared from strain FGSC A4 grown on MM + CA medium for defined periods of time with restricted aeration and in the absence of light. Samples were harvested 12, 24, 48 and 96 h post induction, and extractions were carried out to yield an RNA pool of 1 mg. From this, the pre-amplified custom cDNA library pCNS4 was produced by Invitrogen with a number of 3.2×10^7 primary *E. coli* transformants and an average cDNA insert size of 1.8 kb. As plasmid backbone the vector pCMVSport 6.0 was chosen, into which the cDNA fragments had been ligated directionally via EcoRI and NotI to be flanked by SP6 and T7 oligonucleotide priming sites. Bacterial pools grown from the pCNS4 library stock were plated on LB medium supplemented with Carbenicillin (50 μg ml^{-1}) and colonies were filter-lifted on nitrocellulose membranes. After denaturation and renaturation steps, the bacterial DNA was cross-linked to the membrane by baking and UV exposure. Hybridization with radioactively labelled cDNA probes derived from RNA samples of vegetative FGSC A4 cultures resulted in autoradiographies, from which non-hybridizing bacterial colonies were traced back to their source. In total, 6000 colonies were screened to yield 300 primary candidates that did not hybridize with the vegetative probe pool. This collection of plasmids was screened further with probes derived from asexually sporulating cultures of FGSC A4 and by inverse Southern hybridizations to validate differential expression of the respective transcript during cleistothecia formation of *A. nidulans*.

Plasmid constructions

Plasmids used and constructed during the course of this study are listed and briefly described in Table 2. Sequences of oligonucleotides employed in cloning and manipulation of nucleic acids are given in Table 3. More precisely, plasmids of this study were generated as follows: pME3020, which carries a full-length *grrA* cDNA, was constructed by combining the 1.4 kb amplicon that resulted from a PCR with primers Sv126/128 on pCNS4 as EcoRV/SpeI fragment with the cDNA insert of pME3019 transferred as EcoRV/NotI fragment into pBluescript II KS. The genomic *grrA* locus was isolated in form of plasmid pME3023 as 8.7 kb HpaI/NheI fragment in

Table 2. Plasmid constructs utilized in this study.

Plasmid	Description and characteristics	Reference
pBluescript II KS	General cloning plasmid (*bla*, multiple cloning site)	Stratagene
pCMVSport 6.0	pCNS4 library plasmid (*bla*, ^{P}*CMV*)	Invitrogen
pPTR II	Autonomously replicating *Aspergillus* plasmid (*ptrA*, *AMA1*, *bla*)	Takara
pMCB32	^{P}*alcA*::sGFP(S65T) expression plasmid	Fernandez-Abalos *et al*. (1998)
pME3019	pCNS4 EST no. 5, contains partial *grrA* cDNA	This study
pME3020	Complete *grrA* cDNA in pBluescriptII KS (EcoRV/NotI)	This study
pME3021	^{P}*MET25::grrA::CYC1*^{t}, *URA3*, *ARS/CEN* yeast low-copy expression plasmid	This study
pME3022	^{P}*MET25::grrA::CYC1*^{t}, *URA3*, 2 μm yeast high-copy expression plasmid	This study
pME3023	Genomic HpaI/NheI *grrA* fragment (8.7 kb) in pBluescriptII KS EcoRV/SpeI	This study
pME3024	*A. oryzae ptrA* resistance gene flanked by SfiI sites in pBluescript II KS	This study
pME3025	*grrA::ptrA* gene replacement cassette	This study
pME3026	*A. fum. pyrG* as 1.9 kb EcoRI fragment in pBluescript II KS	This study
pME3027	^{P}*grrA::GFP-GA*$_5$ *-grrA* expression module in pME3026	This study
pME3028	^{P}*gpdA::GFP-GA*$_5$ *-grrA* expression module in pME3026	This study

pBluescript II KS (EcoRV/SpeI), identified by colony hybridization screening a partial library that had been constructed from FGSC A4 genomic DNA. The *ptrA*(SfiI)-containing plasmid pME3024 was generated by amplification of the *A. oryzae* resistance gene *ptrA* (Kubodera *et al*., 2000) from pPTR II (Takara) with primer pair Sv199/200 and inserting the resulting 2 kb amplicon into pBluescript II KS opened by EcoRV digestion. The *grrA::ptrA* deletion cassette was constructed adapting the protocol of Kämper (2004) as follows: 5′ and 3′ regions flanking the *grrA* coding sequence were amplified from genomic DNA of wild-type strain FGSC A4 with primer pairs Sv201/203 and Sv202/204 to result in a 2 kb amplicon each. As incorporated by the priming oligonucleotides, both carry a SfiI restriction site at one end, which are not compatible because of the unique non-palindromic core sequence. From pME3024 the *ptrA* resistance gene was released by SfiI digestion to yield a marker fragment that carries compatible overhangs to either one of the SfiI sites that had been incorporated into the flanking regions. Fragments were mixed in a 1:1:1 ratio to be ligated, and after separation by agarose gel electrophoresis a 6 kb fragment was recovered. Insertion of this directionally ligated product into the cloning vector pBluescript II KS resulted in plasmid pME3025, from which the replacement cassette can be released by BglII/StuI digestion. Yeast expression plasmids pME3021 and pME3022 were constructed by inserting the *grrA* cDNA from pME3020 into derivatives of p416MET25 and p426MET25 (Mumberg *et al*., 1994; Krappmann *et al*., 1999), respectively, via SalI and NotI. GFP-tagged alleles of *grrA* were constructed by single-joint PCR (Yu *et al*., 2004) using oligonucleotides Sv304/308 to amplify the sGFP(S65T) allele from plasmid pMCB32 (Fernandez-Abalos *et al*., 1998) and Sv307/T7 to amplify the *grrA* coding sequence from pME3020. Both amplicons were used as template in a fusion PCR, from which the complete *sGFP-GA*$_5$ *-grrA* module (2.8 kb) was amplified with nested primers Sv305/306. This was inserted into pME3026, which carries the *Aspergillus fumigatus pyrG* marker gene, together with fragments comprising the constitutive *gpdA* promoter (1.7 kb SspI/ScaI) or the native *grrA* promoter (2.0 kb FspI/BglI).

Cytological staining and microscopy

Cleistothecial contents were stained with acriflavin (FLUKA No. 01673) according to the protocol of Swart *et al*. (2001) and samples were mounted in 50% glycerol/10% phosphate buffer (pH 7)/0.1% n-propyl-gallate. Cells were examined with a Zeiss Axiovert microscope by either differential interference contrast microscopy (DIC) or fluorescence microscopy using a GFP filter set (AHF Analysentechnik AG, Tübingen, Germany). Photographs were taken using a Xillix Microimager digital camera and the Improvision Openlab software (Improvision, Coventry, UK).

Table 3. Oligonucleotides used for plasmid constructions.

Designation	Sequence
Sv126	5′-AGT CAG ATC AAG AAT GCG AAG AC-3′
Sv128	5′-TTC GGC TGC AAA AGC CTA CGC AGC-3′
Sv199	5′-GGC CAC TCA GGC CAA TTG ATT ACG GGA TCC CAT TGG-3′
Sv200	5′-GGC CTA GAT GGC CTC TTG CAT CTT TGT TTG TAT TAT AC-3′
Sv201	5′-TGC GCG GGA CTC ACT ACT GCC TGC-3′
Sv202	5′-AAC TTA GTG AGC AAG TAC GCC AGG-3′
Sv203	5′-TAG GCC TGA GTG GCC GAT GGC GGC GTT GAG GTT GGG CTG C-3′
Sv204	5′-TAG GCC ATC TAG GCC GGC TCC CCC CGA GTT CAC CCG ACA GC-3′
Sv304	5′-TCG ACG GTA TCG ATA AGC TTG ATC C-3′
Sv305	5′-ATC ATG GTG AGC AAG GGC GAG GAG C-3′
Sv306	5′-GGG TAT AGA ATC TTC ATA TGT GCC ATC G-3′
Sv307	5′-GGA GCT GGT GCA GGC GCT GGA GCC GGT GCC CGT TCT CGG CAG CCA ACT CGA TTC TCG-3′
Sv308	5′-GGC ACC GGC TCC AGC GCC TGC ACC AGC TCC CTT GTA CAG CTC GTC CAT GCC GTG AGT G-3′

Acknowledgements

We thank Verena Große for excellent technical assistance and all other members of the department for inspirational discussions and helpful support. We are indebted to Kap-Hoon Han for valuable advice in the initial phase of the project during a research visit of S.K. at the Oklahoma State University, supported by a Travel Allowance Scholarship from the Boehringer Ingelheim Fonds. Anne Obermayer is thanked for assistance with microscopy studies. Funding was received from the Deutsche Forschungsgemeinschaft, the Volkswagen Stiftung, and the Fonds der Chemischen Industrie. Moreover, we thank Nigel Dunn-Coleman, Genencor International, and the National Science Foundation (NSF 98-13360) for their support.

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